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Research Article

Vulpinic Acid Targets WNT/β-Catenin Signalling Pathway in HeLa Cells

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Abstract

Cervical cancer is one of the most common cancers in women. Due to the side effects and inadequate treatment methods of current cancer drugs used in the treatment of cervical cancer, it is important to develop new treatment strategies. In our previous study, we reported that vulpinic acid (VA), a natural lichen secondary metabolite with remarkable biological activities, exhibited anti-proliferative, apoptotic, and anti-migratory properties in cervical cancer HeLa cells, and the IC_{50} value of VA was calculated as 66.53 µg/mL at 48 h. However, the effect of VA on the WNT/β-catenin signaling pathway, which plays a role in various biological processes including tumorigenesis, cell proliferation, cell cycle regulation, embryogenesis, metastasis, cellular differentiation, apoptosis and drug resistance, is unknown. In this study, we aimed to elucidate whether VA exerts its anti-migratory effect on HeLa cells treated with IC_{50} dose through the WNT/β-catenin signalling pathway. In summary, this study demonstrated that the suppression of migration of HeLa cells by VA may be mediated by inhibition of the WNT/β-catenin signalling pathway. VA may be a natural active compound candidate for the therapy of human cervical cancer and may be among the inhibitory candidates of the WNT/β-catenin signalling pathway.

Keywords: lichen secondary metabolite, anti-migratory effect, gene expression

Vulpinik Asit HeLa Hücrelerinde WNT/β-Catenin Sinyal Yolağını Hedefler

Öz

Rahim ağzı kanseri kadınlarda en sık görülen kanserlerden biridir. Rahim ağzı kanseri tedavisinde kullanılan mevcut kanser ilaçlarının yan etkileri ve yetersiz tedavi yöntemleri nedeniyle yeni tedavi stratejilerinin geliştirilmesi önem arz etmektedir. Önceki çalışmamızda, önemli biyolojik aktiviteye sahip doğal bir liken sekonder metaboliti olan vulpinik asidin (VA), serviks kanseri HeLa hücrelerinde anti-proliferatif, apoptotik, ve anti-göç özellikleri sergilediğini ve VA'nın IC_{50} değerini 48 saatte 66.53 µg/mL olarak hesaplandığını bildirdik. Fakat, VA'nın, tümörigenez, hücre proliferasyonu, hücre döngüsü düzenlemesi, embriyogenez, metastaz, hücresel farklılaşma, apoptoz ve ilaç direnci gibi çeşitli biyolojik süreçlerde rol oynayan WNT/β-katenin sinyal yolağı üzerindeki etkisi bilinmemektedir. Bu çalışmada, VA'nın IC_{50} dozu ile tedavi edilen HeLa hücreleri üzerindeki anti-göç etkisini, WNT/β-katenin sinyal yolağı üzerinden gösterip göstermediğini aydınlatmayı amaçladık. Özetle, bu çalışma HeLa hücrelerinin göçünün VA tarafından baskılanmasına WNT/β-katenin sinyal yolağının inhibisyonunun aracılık edebileceğini göstermiştir. VA, insan serviks kanserinin tedavisi için doğal bir aktif bileşik adayı olabilir ve WNT/β-katenin sinyal yolağının inhibitör adayları arasında yer alabilir.

Anahtar Kelimeler: liken sekonder metabolit, anti-göç etki, gen ekspresyonu

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1. Introduction

Lichen, composed of one type of algae (cyanobacteria) and one type of fungus, produce secondary metabolites with various chemical structures, including terpenes, aromatics, cycloaliphatics, and aliphatics (1,2). Lichen secondary metabolites have numerous bioactivities such as anti-inflammatory, antiviral, antibiotic, antioxidant and anticancer (3,4). Recently, an increasing amount of data suggests that lichen metabolites may exhibit cytotoxic, anti-migratory, and apoptotic effects on cancer cells and may interfere with critical signaling pathways in cancer (5–7). These metabolites may enhance the anticancer activity of chemotherapy and radiotherapy and contribute to improving the side effects of existing drugs. Therefore, identifying new anticancer agents in targeted cancer therapies and understanding their molecular targets are important in the research and development of effective treatment methods.

Cervical cancer is one of the most common cancers in women and the discovery of new adjuvant agents and new treatment strategies is extremely important due to the side effects and inadequate treatment methods of current cancer drugs used in treatment (8). In particular, the potential of anticancer agents to inhibit cancer-related signaling pathways and induce apoptosis constitutes an interesting research area in cancer treatments (9,10).

WNT/β-catenin has been implicated in many biological process, including tumorigenesis, cell proliferation, cell cycle regulation, embryogenesis, metastasis, cellular differentiation, apoptosis, and drug resistance (11). While this pathway is not active in normal cells, it is dysregulated and abnormally activated in neurological diseases, inflammation and cancer (12). The main transcriptional co-activator of the WNT signaling pathway is β-catenin, which is controlled by a degradation complex consisting of casein kinase 1 (CK1), glycogen synthase kinase 3β (GSK-3β), axis inhibition protein (AXIN) and adenomatous polyposis coli (APC). WNT binds to Frizzled (FZD) and its co-receptor Lipoprotein Receptor Related Protein (LRP) on the cell membrane surface in the presence of WNT ligands. This induces phosphorylation of LRPs recruiting Dishevelled (DVL) and AXIN and allows degradation of the destruction complex (13). After cytoplasmic accumulation, β-catenin translocates to the nucleus and interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) to induce transcriptional activation of its target genes (14). Therefore, inhibition of the WNT/β-catenin pathway in cancer cells has been highlighted as a potential target for cancer treatment, as it suppresses metastasis and tumor progression (14,15).

Vulpinic acid (VA), a secondary metabolite isolated from lichens such as *Letharia columbiana*, *Letharia vulpina*, *Pseudocyphellaria flacicans*, and *Vulpicida pinastri*, has antibiotic and anticancer activities (5,16–18). In our previous study, cytotoxic, apoptotic, and anti-migratory effect of VA, was demonstrated in cervical cancer HeLa cells (19).

However, it is not known whether the WNT/β-catenin signaling pathway mediates this anti-migratory effect of VA in HeLa cells. The aim of this study was to decipher the potential effect of VA on the WNT/β-catenin pathway and its target genes.

2. Material and Methods

2.1. Cell Culture and Treatment Conditions

The human cervical cancer cell line (HeLa) was obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium (Gibco™). This medium contained 1% penicillin/streptomycin (Sigma-Aldrich), 1% L-glutamine (Thermo Fisher Scientific), and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone), and the cells were cultured at 37°C in a 5% CO_2 atmosphere.

2.2. Preparation of VA

VA ($C_{19}H_{14}O_5$, Cayman Chemical Company) was prepared as a stock solution in dimethyl sulfoxide (DMSO, Sigma). It was stored at -20°C until needed.

2.3. Total mRNA Extraction

In the previous study, the anti-proliferative effect of VA in HeLa cells was investigated in a dose (0-100 µg/mL) and time (24 and 48 h) dependent manner, and its effective IC_{50} dose (50% inhibition concentration) for 48 h was determined as 66.53 µg/mL (19).

HeLa cell line was seeded at 2 mL (150.000 cells/mL) per well of a 6-well plate and incubated at 37°C in a 5% CO_2 atmosphere. The medium was then removed and the wells were washed with Dulbecco's phosphate buffered saline (DPBS) (Gibco™) buffer. Fresh complete medium was added to the control group. The treatment group was treated with VA (IC_{50} concentration at 48 h) determined in fresh complete medium in triplicate and cells were incubated at 37°C in 5% CO_2 atmosphere. The medium was aspirated and the wells were washed with DPBS. To harvest cells from the wells, 600 µL lysis buffer (10 µL β-mercaptoethanol per 1 mL lysis buffer) was added to each well. RNA was isolated according to the manufacturer's protocol using the PureLink™ RNA Mini Kit (Invitrogen). The purity and concentrations of total RNA samples isolated from cells were determined by microplate reader (Thermo Scientific Multiscan GO). RNA concentrations were equalized to ensure equal experimental conditions (19–21).

2.4. cDNA Synthesis

cDNA was synthesized from total RNA obtained from the cells using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol (22).

2.5. Analysis of Gene Expression by Quantitative Real-Time PCR (qPCR)

The expression levels of *WNT2, AXIN1, DVL1, CTNNB1 (β-catenin), TCF4, CDK1, c-MYC*, and *CCND1* genes were measured by qPCR using SYBR Green Master Mix (Qiagen). *β-*

ACTIN was used as a housekeeping gene. To the qPCR reaction mixture; cDNA (100 ng-100 fg), 0.3-0.5 µM of each primer, 10 µL of Syber Green master mix were added and the total volume was made up to 20 µL with nuclease free water. The reaction conditions were set to include a denaturation step at 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and ≤60°C for 10 seconds. Table 1 shows the base sequences of the primers used in qPCR experiments. An analysis of the amplification curve was performed to measure the amplified products. Experiments were repeated in triplicate. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method (23).

Table 1. The list of primers used in this study.

Primers	Sequence (5'—3')
WNT2	Forward- AAGGAAAGGATGCCAGAGCC
	Reverse- TGCACATCCAGAGCTTCCAG
AXIN1	Forward- CGTCTGGAGGAGGAAGAAAAGAG
	Reverse- CTCTGCGATCTTGTCTCTGTCT
DVL1	Forward- GATGGACAACGAGACAGGCA
	Reverse- CGGCATCGTCATTGCTCATG
CTNNB1(β-catenin)	Forward- GCTTGGTTCACCAGTGGATT
	Reverse- GTTGAGCAAGGCAACCATTT
TCF-4	Forward- GAGGCCAAGGTTTGTGTGAT
	Reverse- CACTGCTCACAGGAGGTGAA
CDK1	Forward- GGCTCTGATTGGCTGCTTTG
	Reverse- GGTAGATCCGCGCTAAAGGG
c-MYC	Forward- TACAACACCCGAGCAAGGAC
	Reverse- GAGGCTGCTGGTTTTCCACT
CCND1	Forward- GGCGGAGGAGAACAAACAGA
	Reverse- CTCCTCAGGTTCAGGCCTTG
β-ACTIN	Forward- TGCTATCCCTGTACGCCTCT
	Reverse- CTCCTTAATGTCACGCACGA

2.6. Statistical Analysis

Data are presented as mean±SD from three experiments. The unpaired t-test was performed to statistically compare the results in GraphPad Prism (GraphPad Software version 5.0 for Windows). An asterisk (*) indicates statistically significant changes. Symbols are defined below: $p>0.05$ (not significant, ns); $*p<0.05$ (significant), $**p<0.01$ (highly significant), and $***p<0.001$ (extremely significant).

3. Results and Disscussion

In our previous study, VA was shown to have dose (0-100 µg/ml) and time (24 h and 48 h) dependent cytotoxic potential in HeLa cells by XTT assay, and the IC_{50} value was calculated as 66.53 µg/mL at 48 h. It was also reported that VA significantly suppressed migration by Transwell migration assay and induced apoptosis by flow cytometry in HeLa cells treated

with the IC_{50} value (19). However, the inhibitory mechanism of VA on HeLa cell migration is not stil known. This study investigated whether VA interferes with Wnt/β-catenin signaling, which has been identified as a therapeutic target in many cancers, including cervical cancer. For this purpose, the effect of VA on WNT pathway in HeLa cells was evaluated by qPCR. The findings indicated that the VA exhibited a tendency to reduce the gene expression of WNT2, a crucial component in the activation of the specified pathway, but it was not statistically significant ($p>0.05$). (Figure 1A). In the literature, it is known that *WNT2* is overexpressed in cervical cancer, colorectal cancer, pancreatic cancer, human fibroadenomas, and breast cancer and thus triggers migration and invasion (24–30). Considering the function of WNT2 in this pathway, the downward trend in its expression may be due to the inhibitory effect of VA on the pathway.

DVL1 mRNA levels, a positive regulator of the pathway, was repressed by VA in HeLa cells ($p<0.01$) (Figure 1B). *AXIN1* gene expression, an essential component of the degradation complex, was significantly increased in VA-induced HeLa cells ($p <0.001$) (Figure 1C). Given that AXIN1 negatively regulates the signaling pathway by inducing CTNNB1 degradation, the observed increase in *AXIN1* may mean that the destruction complex is activated (31). As for CTNNB1, a trend towards down-regulation was observed in VA-induced HeLa cells, but without statistical significance ($p>0.05$) (Figure 1D).

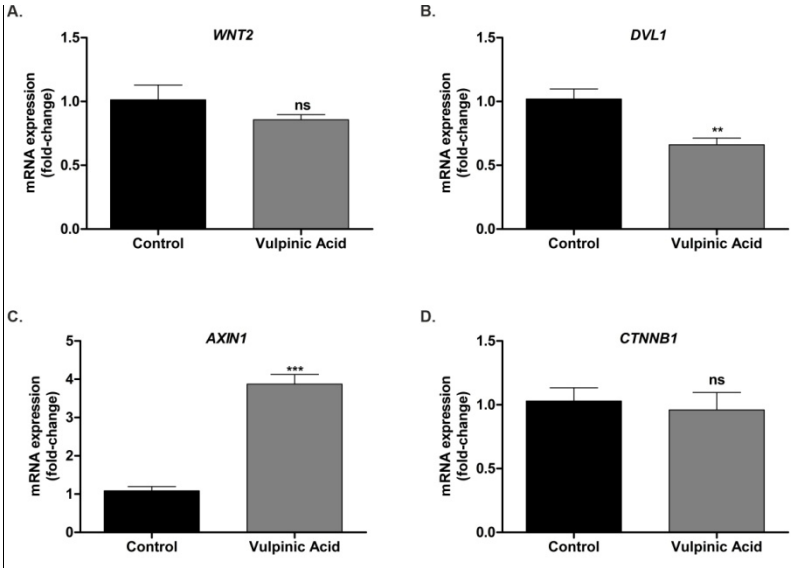

Figure 1. Effect of VA on the gene expression levels of the WNT/β-catenin pathway. **A-D** Expressions of *WNT2, DVL1, AXIN1*, and *CTNNB1* genes analyzed by qPCR in HeLa cell line.

A decrease in the mRNA level of the transcription factor *TCF-4* was observed in VA-treated HeLa cells ($p<0.001$) (Figure 2A). TCF4 binds to DNA following CTNNB1 localization to the nucleus and regulates transcription of several target genes such as *c-MYC*, *CCND1*, and *CDK1* (32). It has been hypothesized that the decrease in *TCF-4* may also affect the expression of WNT signaling pathway target genes that promote cell proliferation, cell death and metastasis. The results showed that *c-MYC*, an oncogene, was significantly decreased by VA treatment ($p<0.01$) (Figure 2B). Interestingly, VA did not affect *CCND1* expression

($p>0.05$), while *CDK1* expression ($p<0.001$) was increased (Figure 2C-D). Overexpression of these target genes plays critical roles at cell cycle transition points and promotes the process of oncogenesis. It is thought that VA may inhibit proliferation, especially through *c-MYC*. As for the increase in *CDK1*, this suggests that cancer cells may have increased the expression of this gene as an adaptive response to develop resistance to the anticancer agent or to compensate for its effect.

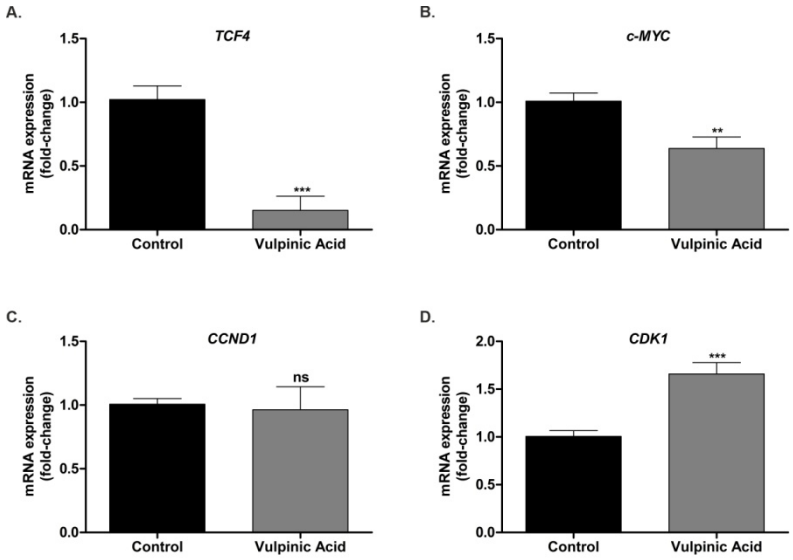

Figure 2. Effect of VA on the expression of TCF-4 and WNT signaling target genes including *c-MYC*, *CCND1*, and *CDK1* after 48 h treatment in HeLa cell line.

Natural products play an important role in various cancer therapies by regulating the WNT/β-catenin pathway (33). In the literature, caperatic acid and physodic acid, which are secondary metabolites of lichen, exhibit anticancer properties by inhibiting the WNT/β-catenin pathway in colorectal cancer (34). Atranorin suppressed β-catenin and regulated downstream target genes such as *c-MYC, CCND1,* and *CD44* in lung cancer (35). The WNT/β-catenin pathway has been shown to be down-regulated in glioblatoma multiforme cells by treatment with caperatic acid alone or in combination with tamoxifen (36). Curcumin treatment has been reported to suppress the proliferation of colon cancer by inhibiting the WNT/β-catenin pathway in mice (37). Another study reported that ursolic acid and corosolic acid, natural pentacyclic triterpenoids derived from various plants, inhibited the WNT/β-catenin pathway in colon cancer cells (38).

Taken together, our findings suggest that VA inhibits the WNT pathway by activating the β-catenin degradation complex (Table 2). This study demonstrated that the anti-proliferative and anti-migratory effects of VA may be mediated by the inhibition of WNT/β-catenin signalling in HeLa cells.

Table 2. VA-regulated mRNA levels of WNT/β-catenin signaling in HeLa cells

Gene	qPCR
WNT2	↔
AXIN1	↑
DVL1	↓
CTNNB1(β-catenin)	↔
TCF-4	↓
c-MYC	↓
CCND1	↔
CDK1	↑

4. Conclusion

To summarize, this study showed that for the first time that VA treatment in cervical cancer suppresses migration by inhibiting the WNT/β-catenin pathway mechanism. Overall, VA may be a promising inhibitor candidate for cervical cancer therapy by targeting the Wnt/β-catenin signaling pathway.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Designing the study, ŞNK; collecting the data, ŞNK; evaluating the results, ŞNK; writing the article, ŞNK.

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